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Active

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Center # : R5983-7A0 Center shr #: OCA file #:
Work type : RES
Contract#: 5 R01 HL28167-07 Mod #: Document : GRANT
Prime #: Contract entity: GTRC

Subprojects ? : N
Main project #:

Project unit: CHEM Unit code: 02.010.136
Project director(s):
MAY S W CHEM

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Award period: 880701 to 890630 (performance) 890930 (reports)

Sponsor amount	New this change	Total to date
Contract value	209,855.00	209,855.00
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Title: NOVEL ANTIHYPERTENSIVES: RATIONAL DESIGN AND EVALUATION

PROJECT ADMINISTRATION DATA

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Security class (U,C,S,TS) : ONR resident rep. is ACO (Y/N): N
Defense priority rating : supplemental sheet
Equipment title vests with: Sponsor GIT X
NO EQUIPMENT MAY BE PURCHASED IN LAST 6 MONTHS OF THIS GRANT YEAR (12/30/88).

Administrative comments -
INITIATION. 7TH YR OF GRANT(TOTAL PROJECT PERIOD: 01/01/82 THROUGH 06/30/88)

GEOP A INSTITUTE OF TECHNOLOGY
OFFIC. OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 04/02/90

Project No. G-33-W07 Center No. R5983-7A0

Project Director MAY S W School/Lab CHEM

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH

Contract/Grant No. 5 R01 HL28167-07 Contract Entity GTRC

Prime Contract No.

Title NOVEL ANTIHYPERTENSIVES: RATIONAL DESIGN AND EVALUATION

Effective Completion Date 890630 (Performance) 890930 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	Y	
Final Report of Inventions and/or Subcontracts	N	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other	N	

Comments CONTINUED BY G-33-W08

Subproject Under Main Project No.

Continues Project No.

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	N
Reports Coordinator (OCA)	N
GTRC	Y
Project File	Y
Other	N
	N

A. SPECIFIC AIMS

To date, we have developed several classes of compounds which we have shown to be either: (a) novel, potent substrate analogs for dopamine beta-monooxygenase (DBM) capable of competing with dopamine for enzymatic oxygenation; (b) substrates which cause a DBM-turnover-dependent *local* ascorbate depletion cycle to occur; (c) mechanism-based irreversible inhibitors or reversible inhibitors of DBM. For several of our compounds we have demonstrated a potent antihypertensive activity, and we hypothesize that this activity arises via a modulation of adrenergic neuronal activity mediated through DBM oxygenation. The broad objectives of our program are to confirm key aspects of the biochemical and pharmacological mechanism responsible for the antihypertensive activity of compounds in hand, and to extend our efforts by continuing the design, synthesis, and evaluation of other novel compounds potentially capable of modulation of adrenergic neuronal activity.

Selenium-Containing Compounds: The unique aspect of this class of compounds arises from our hypothesis that they cause modulation of adrenergic neuronal activity through the novel strategy of turnover-dependent *local* depletion of the essential DBM cofactor, reduced ascorbate ($ASCH_2$). Because of this unique mechanism, these compounds may have wide ranging applications in neuroscience -- in addition to their potential as hypotensive agents -- so it is critical that we confirm our hypothesized mechanism of action. We will demonstrate this process directly in cell culture, show correlation between the enantioselectivities of enzymatic turnover and $ASCH_2$ depletion even in the presence of fully functional $b561$ recycling system, probe whether we can "fine tune" turnover-dependent recycling -- in a kinetic sense --by judicious choice of the particular selenide substrate, and determine effective tissue concentrations for correlation with isolated tissue experiments.

Sulfur-Containing Compounds: This class represents our simplest strategic approach to the modulation of adrenergic neuronal activity. We will demonstrate that the key elements of the mechanism are operative in functioning cells in culture, carry out pharmacodynamics to confirm a primarily *peripheral* locus of action and minimal CNS access for hydroxylated derivatives (a key consideration in any eventual clinical utilization of hydroxy derivatives of many of our various classes of compounds), and correlate the enantioselectivity of DBM sulfoxidation and effects in PC-12 cell culture and granule ghosts.

Optimized Suicide Substrates: We will draw one our extensive kinetic and mechanistic enzymological characterization of our "olefinic" and "N-dealkylation" mechanism-based inhibitors wherein we have developed compounds which are *rapid* inactivators with *very low partition ratios* (as low as unity) but still maintain structural features necessary for solubility and presynaptic uptake. Thorough bioassays in SHR as well as companion experiments to demonstrate cellular and subcellular uptake, enzymatic oxygenation, and effects on adrenergic outflow will be carried out.

New DBM-Targeted Compounds: Five new classes of DBM-targeted compounds, which we reason will be potent suicide substrates, prodrugs, pseudosubstrates or high affinity non-covalent inhibitors, will be synthesized and fully characterized as to enzymology. Those compounds whose structural features, chemical properties and enzymological parameters are most promising will be bioassayed in SHR and examined at the cellular and subcellular levels.

Tyrosine Hydroxylase (TH): We will carry out a very limited pilot evaluation of one class of compounds targeted at the enzyme TH, in order to provide a measure of the importance of the sequestering and uptake accessibility of DBM in terms of rationale drug design. Also, if TH is indeed the rate limiting step in the NE biosynthesis pathway, we may obtain insight into the consequencesof "DBM-targeted modulation" vs more drastic "TH-targeted cutoff" on NE production. The specificity demands of TH for an amino acid structure will limit these experiments to the enzyme and cellular levels, and *no bioassays* in SHR are planned.

DBM Mechanism: We will continue our contributions to the mechanistic enzymology of DBM by demonstrating DBM-catalyzed aromatization of compounds designed to trap reaction transients generated by single electron transfer and through active site peptide mapping with one of our suicide inhibitors which is ideal for this purpose.

BACKGROUND AND SIGNIFICANCE

Cardiovascular diseases are the leading cause of premature mortality in industrialized nations. Hypertension of unknown etiology may be the major causative factor in the genesis of these diseases (1). Traditionally, hypertension has been treated clinically by employing agents which either: (a) are

antagonists at various classes of adrenergic receptors; (b) modify renal function (to induce diuresis, for example); (c) act directly on smooth muscle (vasodilators); (d) act as neuronal blockers (reserpine, guanethidine); (e) block calcium channels in vascular musculature; or (f) induce alterations in the renin-angiotensin system (captopril, saralasin, and the like). In recent years, research has been focused in the main on the newest of these approaches (calcium blockers, renin-angiotensin inhibitors) while considerable progress has been made in the design and testing of adrenergic receptor ligands which are more specific for particular receptor sub-populations or discrete anatomical regions (for example, centrally-active agents such as clonidine or propranolol). A more complete discussion of the treatment of hypertension may be found in several very good reviews (2,3).

We have visualized a possible new approach to the control of hypertension which focuses on an important enzyme of catecholamine synthesis: dopamine beta-monooxygenase (EC 1.14.2.1, DBM). DBM is a copper-containing enzyme which catalyzes the terminal oxygenation in the synthesis of the principal adrenergic neurotransmitter, norepinephrine (NE). It has long been recognized that the sympathetic nervous system, and its neurotransmitter NE, are responsible for regulation of vascular tone and the physiology of hypertension (4). Together with data correlating increased NE levels with increased blood pressure (5) and with the occurrence of congestive heart failure (6), these facts support a rationale for developing compounds which can interfere with NE biosynthesis as potential agents for the treatment of hypertension and congestive heart failure.

In considering such an approach, we immediately recognized two significant advantages of focusing on DBM as our target enzyme. First, DBM is an enzyme which is physiologically functional only within adrenergic neurotransmitter storage vesicles (and their adrenal homologues, the chromaffin granules). This sequestration of DBM away from the normal cytosolic flux, and its intimate compartmentalization with its substrate, product and requisite cofactors, places it in a metabolically convenient position for chemical and pharmacological manipulation (e.g., with our selenides which deplete *local* reduced ascorbate; see below). Second, and perhaps most significantly, it is well known that termination of adrenergic "firing" is accomplished by the presynaptic reuptake of NE (and its at least partial re-storage in vesicles). This provides a pathway by which our novel chemical agents could gain access to -- and modify the activity of -- DBM, so long as we took care to design compounds possessing those structural features essential for presynaptic reuptake. We considered this latter point highly attractive, since a major concern in enzyme-targeted drug design is always "How does one target one's novel compounds for the enzyme of interest?"

There are additional considerations relevant to the rationale of our approach of designing molecules targeted at DBM as potential cardiovascular agents. There are four steps in the pathway leading from the essential amino acid, Phe, to the neurotransmitter, NE. [Of these, the tyrosine hydroxylase (TH)-catalyzed hydroxylation of tyrosine to dihydroxyphenylalanine was regarded as the putative rate limiting step in the early literature (7), although, as pointed out later (8), there may be a shift toward rate determining DA hydroxylation under certain metabolic conditions.] In any case, since DBM is an "internal enzyme" in the catecholamine cascade, an approach aimed at interfering with DBM activity may offer the advantage of a diminished probability for enzymatic "upregulation" -- with its attendant tachyphylaxis -- upon chronic administration of the drug (9). A second point is that interfering with DBM may lead to a buildup of DA, a molecule known to have vasodilator action, particularly in the renal and mesenteric vasculature. This is an area in which our collaborator, Professor Leon Goldberg, is an acknowledged international authority (see Collaborative Arrangements). Finally, it should be mentioned that if, indeed, TH and not DBM is rate determining step in catecholamine biosynthesis, then interfering with DBM may cause modulation, rather than virtual shutdown, of adrenergic outflow. This would be of particular interest to us since it has been long presumed that many of the side effects of classical receptor antagonists is due to their completely "turning off" adrenergic outflow, as opposed to such a more gentle modulation.

During the course of this project we have elaborated several novel classes of DBM-targeted compounds, the oxygenation of many of which represented a heretofore unrecognized activity for DBM. Accordingly, in each case, we first had to unequivocally demonstrated that prototypes of each class are indeed substrates and/or turnover-dependent inhibitors of DBM. While our ultimate goal is obviously to produce compounds with attractive pharmacological properties, our approach has been to first carefully define the enzymology of these compounds and the biochemistry of their interaction with DBM.

Correspondingly, we have carried out bioassays to demonstrate cardiovascular activity of our compounds, and a variety of experiments at the tissue, cellular, and subcellular level to establish a link between the enzymology of our compounds as DBM substrates and/or inhibitors and a modification of sympathetic tone leading to antihypertensive activity. By way of background, the following examples illustrate the approaches we have followed in the design of DBM-targeted compounds (Background details can be found in references 10-16).

Our initial goal was to examine the reactivity of DBM toward functionalities other than benzylic C-H bonds; prior to our work, DBM had been viewed as an absolutely "specific hydroxylase". In the first demonstration of an alternative oxygenation competence by any of the so-called "specific hydroxylases", we reported that DBM oxygenates phenyl 2-aminoethyl sulfide (PAES) to the corresponding sulfoxide, phenyl 2-aminoethyl sulfoxide (PAESO). We examined a number of derivatives of PAES, and demonstrated that both the parent compound and its ring substituted and/or alpha-methylated derivatives are excellent substrates for DBM and are oxygenated to the corresponding sulfoxides via the "normal" reductive oxygenation pathway of the enzyme. Sulfoxidation of our sulfide substrates is kinetically facile, proceeding more rapidly than methylene hydroxylation in cognate substrates. Moreover, sulfoxidation is highly stereospecific, exhibiting the same reaction stereochemistry as the normal hydroxylation reaction.

We found that our sulfide substrates exhibit potent antihypertensive activity in SHR. Both chronic and acute studies have been carried out at various dose levels, using both direct and indirect blood pressure measuring techniques as well as various modes of drug administration. Based on our results we suggested a mechanism whereby the sulfides gain access to intravesicular DBM and are enzymatically sulfoxidated, thus competing with enzymatic oxygenation of DA to NE. Since we have observed that the sulfoxide products are receptor-inactive, the net result is dilution of the NE released at adrenergic nerve terminii with a resultant diminution in sympathetic outflow. Our ongoing research activities are directed at further elucidation of the mechanism of antihypertensive activity of our sulfides. In addition, refined derivatives of PAES with increased potency, enhanced duration of action, and greater peripheral vs central selectivity are being prepared and evaluated.

We next reported the first example of olefinic oxygenation by DBM and demonstrated that this process leads to suicide inhibition by 1-phenyl-1-aminomethylethene (PAME). On the basis of kinetics, stoichiometry, product trapping, activator effects, and pH dependencies, we established a mechanism-based mode of inhibition. Reaction of DBM with 3H-PAME resulted in non-dialyzable incorporation of radiolabel into the enzyme. Thus, it was clear that olefin oxygenation represented a novel approach to suicide inhibition DBM, which might lead to compounds of pharmacological interest. Subsequently, we carried out extensive mechanistic studies with derivatives of PAME, demonstrated that the mechanism of olefin oxygenation involves initial generation of an olefin-radical-cation and it is this species and not the ultimate epoxide product which leads to inactivation. We also synthesized and enzymologically characterized a number of derivatives of PAME, among them p-OH PAME the most potent suicide substrate for DBM theretofore reported, as well as other derivatives with partition ratios especially favorable for pharmacological purposes. In related work, we also discovered that acetylenic compounds (e.g. 1-phenylpropargylamine) are also highly potent suicide substrates for DBM.

Among the other classes of novel DBM-directed compounds we have developed are product enantiomers which are oxidatively ketonized by DBM, and compounds which undergo enzymatic dealkylation. In the first case, we have demonstrated that S-enantiomers of the alcohol products normally formed by DBM (e.g. S-NE; S-octopamine) are readily hydroxylated by DBM to produce gem-diols as the immediate enzymatic products, which exist in solution as the corresponding ketones. Since such S-enantiomers have weak adrenergic activity, while ketone analogs of norepinephrine and epinephrine exhibit a number of potent pharmacological effects, the ketonization activity of DBM may be exploitable for activation of appropriately designed pro-drugs. In fact, we published the suggestion that this ketonization activity is relevant to the bioactivation of norpseudoephedrine and its cognates. In the second case, we discovered that DBM readily carries out N-dealkylation of N-phenyl ethylenediamine (PEDA) and its cognates in an oxygenative reaction which is kinetically comparable to the other oxygenative activities of this enzyme. As detailed in PROGRESS REPORT, during this current project period we have extensively investigated the mechanism of this new N-delakylation activity of DBM, shown that potent,

kinetically well-behaved suicide inactivation occurs concomitant with N-dealkylation, and proposed a unified mechanism for oxygenation of heterooatom and olefinic substrates.

Finally, we designed phenyl-2-aminoethyl selenide (PAESe) as the prototype for a novel class of aminoalkyl phenyl selenide DBM substrates. We anticipated that DBM would carry out selenoxidation with high facility, but recognized that the expected selenoxide products should exhibit unique reactivity and redox properties. We confirmed that PAESe is indeed an excellent substrate for DBM and that enzymatic oxygenation produces phenyl-2-aminoethyl selenoxide (PAESeO), with kinetic parameters comparable to those for oxygenation of other facile substrates.

What is remarkable about the processing of PAESe by DBM is that the enzymatic product, PAESeO, possesses a redox capability unlike that of any other DBM product. We observed that under standard *in vitro* assay conditions, PAESeO is non-enzymatically reduced back to PAESe with the concomitant and stoichiometric oxidation of reduced ascorbate ($ASCH_2$). This non-enzymatic recycling reaction is a facile, stoichiometric process which we thoroughly characterized in our laboratory during this current project period. As detailed in PROGRESS REPORT, we have clearly shown in *in-vitro* experiments that once enzymatic processing of PAESe by DBM begins, newly formed PAESeO is recycled back to PAESe, and this cyclic oxidation/reduction pathway continues until reduced ascorbate is depleted. Since reduced ascorbate is an essential cofactor for DBM, this *in-situ* depletion of reduced ascorbate prematurely terminates enzymatic activity. These findings establish novel biochemistry for the selenide substrate.

Initial examinations of the effects of PAESe on systemic blood pressure *in vivo* were equally striking. We observed that PAESe exhibits a marked antihypertensive effect in SHR, with as much as a 50% reduction in blood pressure. In view of these promising results, it has become a high priority to us to focus on the question of whether the antihypertensive activity we observe for our selenides in SHR indeed arises via the DBM-dependent ascorbate-depletion process so evident in our *in vitro* experiments. Moreover, it is well documented that catecholamine granules, the physiological locus of DBM catalysis, possess a cytochrome b_{561}-dependent $ASCH_2$ recycling system, and it was thus critical for us to demonstrate that the selenide-turnover-dependent $ASCH_2$ depletion indeed occurs even in the presence of this physiological $ASCH_2$ recycling system.

C. PROGRESS REPORT

This project was competitively reviewed by the Bio-Organic & Natural Products Chemistry Study Section (Propsal submitted Oct, 1984) and funded for the period 7/1/85 - 6/30/89.

PUBLICATIONS SINCE LAST COMPETITIVE REVIEW

1. "Facile Steroselective Allylic Hydroxylation by Dopamine-beta-Monooxygenase", S.R. Sirimanne and S.W. May, J.Am.Chem.Soc.,110, accepted, in press (issue of 26 October) (1988).
2. "Effects of Dopamine b-Monooxygenase Substrate Analogs on Ascorbate Levels and Noreinephrine Synthesis in Adrenal Chromaffin Granule Ghosts", K. Wimalasena, H.H. Herman, and S.W. May, J. Biolog. Chem., accepted, in press (1989).
3. "Demonstration of the Antihypertensive Activity of Phenyl-2-Aminoethyl Selenide", S. H. Pollock, H. H. Herman, L. C. Fowler, A. S. Edwards, C. Evans, and S. W. May, J. Pharm. Exp. Therap., 246, 227-234 (1988).
4. "An Investigation of the Adrenergic Uptake Specificity of Phenyl-2-Aminoethyl Sulfides", S. H. Pollock, H. H. Herman and S. W. May, Arch. Internat. Pharmacodyn. Therap., accepted, in press (1988).
5. "Novel Antihypertensives Targeted at Dopamine-b-Monooxygenase: Turnover-Dependent Cofactor Depletion by Phenyl Aminoethyl Selenide.",S. W. May, K. Wimalasena, H. H. Herman, L. C. Fowler, M. C. Ciccarello, and S. H. Pollock, J. Med Chem., 31, 1066-1068 (1988).
6. "Demonstration of the Potent Antihypertensive Activity of Phenyl-2-aminoethyl Sulfides," H. H. Herman, S. H. Pollock, L. C. Fowler and S. W. May, J. Cardiovasc. Pharmacol., 11, 501-510 (1988).
7. "Demonstration of the Ascorbate Dependence of Membrane-Bound Dopamine Beta-Monooxygenases in Adrenal Chromaffin Granule Ghosts", H. H. Herman, K. Wimalasena, L. C. Fowler and S. W. May, J. Biolog. Chem., 263, 666-672 (1988).
8. "Ascorbate Depletion as a Consequence of Product Recycling During Dopamine-B-Monooxygenase Catalyzed Selenoxidation," S. W. May, H. H. Herman, S. R. Roberts, and M. J. Ciccarello, Biochemistry, 26, 1626 - 1633 (1987).
9. "Mechanistic Studies on Dopamine-B-Monooxygenase Catalysis: N-Dealkylation and Mechanism-Based Inhibition by Benzylic Nitrogen-Containing Compounds. Evidence for a Single Electron Transfer Mechanism," K. W. Wimalasena and S. W. May, J. Amer. Chem Soc., 109, 4036 - 4046 (1987)

10. "Interaction of Dopamine-B-Monooxygenase with Substituted Imidazoles and Pyrazoles: Catalysis and Inhibition," S. R. Sirimanne, H. Herman, and S. W. May, Biochemical Journal, 242, 227 - 233 (1987).
11. "Olefin Oxygenation and N-Dealkylation by Dopamine-B-Monooxygenase: Catalysis and Mechanism-Based Inhibition," S. R. Padgette, K. Wimalasena, H.H. Herman, S.R. Sirimanne and S. W. May, Biochemistry, 24, 5826-5839 (1985).
12. "Novel Substrates and Inhibitors for Dopamine B-Monooxygenase: Biochemical and Pharmacological Studies," S.W. May, H.H. Herman, S.R. Padgette, S.F. Roberts and K. Wimalasena, Acta Pharmaceutica Svecica, Suppl., 1985:2, 302-313 (1985)
13. "N-Succimidyl Methoxy Phenylacetic Acid Ester, an Amine-Directed Chiral Derivatizing Reagent Suitable for Enzymatic Scale Resolutions", P.A. Husain, J.E. Colbert, S.R. Sirimanne, D.G. VanDerveer, H.H. Herman and S.W. May, Analyt. Biochem., submitted.

The Following are Published Abstracts (1985-88)

"Novel Antihypertensives Targeted at Dopamine-b-hydroxylase", Abstracts Third Chemical Congress N. America, Toronto (1988)

"Asymmetric Allylic Oxygenation of 2-(1-Cyclohexenyl)Ethylamine by Dopamine-b-Monooxygenase", FASEB J. 47, 578 (1988).

"Novel Antihypertensives Targéted at Dopamine-b-Monooxygenase", FASEB J. 47, 1752 (1988).

"Comparison of the Antihypertensive Activity of Substrate Analogs for Dopamine Beta-Monooxygenase", S.H. Pollock, H.H. Herman, L.C. Fowler, L.M. DeCarlo, and S.W. May, Abstracts Third Annual Symposium on Cardiovascular Research, American Heart Association, Georgia Affiliate, (1988).

"Determination of the Anorectic Activity of Substrate Analogs for Dopamine Beta-Monooxygenase", J.M. Holbrook, S.W. May, H.H. Herman, S.H. Pollock, D. Jones, W.B. Rose, and M. Shelton, Abstracts Third Annual Symposium on Cardiovascular Research, American Heart Association, Georgia Affiliate, (1988).

"Antihypertensive Activity and Ascorbate Depletion via Product recycling by DBM-Targeted Selenides", Fed. Proc. 46, 1940 (1987).

"Mechanistic and Specificity Studies on Dopamine Beta Monooxygenase Catalysis", Fed. Proc. 46, 2043 (1987).

"The Antihypertensive Activity of a Selenium-Containing Analog of Phenylpropylamine", Fed. Proc. 46, 1290 (1987).

"New Catalytic Activities for a Prototypical Non-Heme Iron Monooxygenase", Fed. Proc. 45, 1537 (1986)

"The Antihypertensive Activity of a Selenium-Containing Analog of Phenylpropylamine", S.H. Pollock, H.H. Herman, S.W. May, L.C. Fowler, and A.S. Edwards, Abstracts First Annual Symposium on Cardiovascular Research, American Heart Association, Georgia Affiliate, (1986).

"Mechanistic Studies on Dopamine-b-Monooxygenase Catalysis: N-Dealkylation and Mechanism-Based Inhibition. Evidence for a Single Electron Transfer Mechanism", Fed.Proc. 45, 1537 (1986)

"Dopamine-b-Hydroxylase: New Catalytic Activities and Mechanism-Based Inhibition", Fed. Proc. 44, 1054 (1985)

"Anti-Hypertensive Activity of Novel DBM Substrates" Fed. Proc. 44, 879 (1985).

Personnel

Faculty: Sheldon W. May, Professor of Chemistry (PI) and Heath H. Herman, Research Scientist since start of project.
Technicians: L. Fowler, 2 years; F. Young, 1 year
Postdoctoral Associates: K.W. Wimalasena; A.K. Katopodis
Graduate Students: K. Wimalasena, S, Sirimanne, P. Husain, L. Fowler

Our objectives for this period were:

To thoroughly characterize the pharmacology and antihypertensive activity we had preliminarily observed with compounds from two different classes of DBM substrate analogs (the sulfur-containing and selenium-containing classes) and initiate bioassay of derivatives of additional classes of our DBM substrate analogs (the olefinic class and the N-dealkylating class);

To explore the subcellular mechanism of action of selected compounds by first demonstrating vesicular and cellular uptake and then enzymatic conversion, of our compounds along with a characterization of the attendant biochemical mechanism of action at the subcellular level;

To extend our efforts to other novel classes of compounds.

Substantial progress has been made in all of these areas. We have thoroughly investigated the pharmacology and antihypertensive activity of our two most-studied classes of compounds and have demonstrated both cellular and subcellular uptake and enzymatic oxygenation of these two classes in adrenergic neuronal tissue. In the course of these studies we have obtained important new information which greatly extends and redefines the significance and scope of our strategic approach to the modulation of adrenergic neuronal activity, a strategy which was highly hypothetical at the inception of the current

grant period. We have investigated compounds of a number of additional classes at both the enzymological and whole-animal level, and have greatly extended the scope of our understanding of the enzymological and physiological constraints of this approach. The information we have obtained in these latter studies suggests several new directions for this work which, we feel, greatly enhances the significance and versatility of our program. The following report highlights our progress in each area with an evaluation of the extent to which our initial objectives have been met. We will divide this section into a discussion of each class of compounds for clarity.

Sulfur-Containing Compounds

We have performed a number of pharmacological and neurochemical experiments with the a-methylated and/or ring p-hydroxylated derivatives of phenyl-2-aminoethyl sulfide (i.e. PAES, MePAES, HOPAES and HOMePAES) over the course of the current project period (17). First, we have shown that all four of these sulfides possess significant antihypertensive activity in spontaneously hypertensive rats (SHR). We examined the time course of the cardiovascular activity of each of four derivatives over a 30 min period, a 6 hr period, and in two-week chronic treatment experiments. We determined that in our earlier initial bioassays, the indirect tailcuff method for measurement of systolic pressure was giving erroneous readings. Using an indwelling carotid catheter, we were now able to continuously measure pulsatile blood pressure and show that the transient indirect sympathomimetic effect of these compounds (a measure of neuronal uptake) was strongly affected by the chemical structure of the derivative. Thus, in MePAES, the alpha methyl functionality give rise to a heightened specificity for the adrenergic reuptake receptor. This is important new information which will be helpful in designing the next generation of these compounds.

With the ability to reliably contrast time course of the antihypertensive activity in SHR of each of four derivatives of PAES, we have now succeeded in obtaining evidence that the locus of bioactivity of our sulfides is principally in the peripheral adrenergic nervous system. This has been a basic presumption inherent in our postulated mechanism that the hypotensive activity of the sufides arises from competition with DA for DBM oxygenation resulting in dilution of peripheral sympathetic outflow. We find that the hydroxylated derivatives HOPAES and HOMePAES produced an antihypertensive effect ca. 75% of the size of that produced by the non-hydroxylated derivatives. Further, in as-yet-unpublished study, we have found an anorexigenic property of some of our sulfides which we have shown is unrelated to their antihypertensive activities and is certainly a CNS effect. Strikingly, the anorexigenic activity is restricted to those derivatives lacking a ring hydroxyl, with the ring-hydroxylated derivatives being completely inert as anorexigenic agents. These findings support the view that the hydroxylated derivatives of our sulfides have a much reduced ability to cross the blood-brain barrier, something which we assumed in our previous proposal by analogy with hydroxylated biogenic amines. We therefore feel much more justified in presuming that the similar hypotensive profiles of all four sulfides reflect the fact that there is little, if any, contribution to the antihypertensive activity of these compounds through CNS effects. We also note in this regards that a mechanism which lowers NE levels will cause a lowering of blood pressure only by decreasing peripheral NE stores; in the CNS it is the elevation of NE levels which can cause peripheral blood pressure to drop.

In work designed to specifically examine both the cellular uptake of these derivatives and to explore the adrenergic reuptake specificity of the alpha-methylated derivatives, we have demonstrated (18) that all derivatives gain entrance to adrenergic neurons through the cocaine-sensitive catecholamine reuptake receptor. We find that not only is the uptake specific and pharmacologically-characterizable, but that this uptake seems to be followed by a rapid entrance of the derivatives into the vesicular pool of NE, as opposed to the cytosolic pooling observed with tyramine. This was highly significant, since such a process obviously would render the sulfides readily accessible for oxygenation by our target enzyme, DBM. Furthermore, bypassing cytosolic pooling may explain an apparent lack of MAO sensitivity of the bioactivity of our compounds (using our non-oral administration routes) despite the fact that PAES and HOPAES are good in vitro MAO substrates in our hands.

We next turned to an examination of the ability of these compounds to directly gain entrance into neurotransmitter vesicles and to be oxygenated therein by DBM, and to the biochemical consequences of these processes as they occur *within* vesicles. In a departure from the experiments we had planned to employ for these examinations, we decided to utilize bovine adrenal medullary chromaffin granule ghosts

instead of synaptosomal vesicles. This change in our plans was largely a result of the fact that this latter preparation from chromaffin granules has been extensively studied and characterized in a number of other laboratories over the past few years, with the result that chromaffin granules and chromaffin granule ghosts have become the preparations of choice for uptake studies. Unfortunately, when we began these studies, there was a report in the literature to the effect that the DBM of chromaffin granule ghosts did not utilize ascorbic acid as a cofactor in its catalytic oxygenative activity. This report cast doubt on the physiological significance of the membrane-bound DBM of chromaffin granule ghosts and meant that any experimentation we were likely to perform with this preparation, particularly those experiments involving activation by DBM, would be suspect. In work detailed in a recently-published J.Biol.Chem paper (19) we reinvestigated this phenomenon and demonstrated that chromaffin granule ghost DBM *does, in fact, utilize intravesicular ascorbic acid as a reductant with great facility*. In addition, we define conditions for the preparation and utilization of these ghosts for the study of DBM-dependent processes which is, in itself, a significant contribution to the literature.

With a thorough understanding of chromaffin granule ghost characteristics now in hand, we set out to answer the three critical questions regarding our presumed mechanism of action of our DBM-directed compounds; namely:

Do the sulfur compounds could gain entrance to these ghosts (which are the widely accepted models for adrenergic neurotransmitter storage vesicles)?

Are the sulfides oxygenated intravesicularly by DBM leading to a buildup of sulfoxide product?

Does intravesicular turnover of the sulfide reduce NE production in functioning vesicles by competing for DBM turnover?

In work detailed in the attached J.Biol.Chem. manuscript in press (20) we have demonstrated that PAES is indeed rapidly concentrated within ghosts under pharmacologically-significant conditions. On the basis of reserpine experiments, we find that the uptake of PAES occurs both through the catecholamine transporter and also by passive diffusion of its free base through the granular membrane. Once PAES gains entrance to the ghosts, *it is rapidly oxygenated by DBM and the product sulfoxide, PAESO, builds up in the intravesicular space*. In other experiments in which equimolar concentrations of DA (which is of course the physiological substrate for granular uptake and DBM oxygenation) and PAES competed both for ghost uptake and enzymatic oxygenation by DBM, we showed that PAES was capable of reducing NE synthesis by as much as 50%. All requisite control experiments, such as demonstration that this effect is dependent on active DBM turnover, normal electron flux into the vesicle, etc. are documented in the paper. Thus, these experiments demonstrate conclusively that, at a subcellular level, using an accepted model for adrenergic neuronal neurotransmitter storage vesicles, PAES is indeed capable of reducing NE synthesis through the basic mechanism we have hypothesized.

With these results with chromaffin granule ghosts in hand, we next wished to turn to the more complete system of adrenergic cells in culture in order to demonstrate these basic processes of uptake and enzymatic conversion of our compounds. To this end, in 1987, we established a tissue culture laboratory in our research group. We have developed and adapted literature preparative methods for the primary culture of bovine adrenal chromaffin cells. We are now able to attain homogeneous populations of chromaffin cells in quantities which match those of the best preparations found in the literature, and which remain viable for an extended period (greater than 18 days). In addition, utilizing a starter culture obtained from Dr. Lloyd Greene, a pioneer in the area, we have continuously cultured and passaged the PC12 line of rat pheochromocytoma cells for more than a year. This cell line originated from a tumor of the chromaffin cells of the rat adrenal medulla, and is an excellent and very well-studied model for adrenergic neurons.(21).

We are currently carrying out initial uptake experiments in cell culture. For theses experiments we are using HOPAES and have just completed the synthesis of HOPAESe; these derivatives are detectable using the very sensitive HPLC/electrochemical detection techniques we have worked out in our laboratory. Once the uptake experiments are complete, we will wish to directly demonstrate the effects of our compounds on NE synthesis. An important operational note is that we have now established reliable conditions under which both media ascorbate and oxygen levels can be maintained and measured during the period necessary for experimentation This is a non-trivial point for the types of experiments we wish to do with the sulfides and selenides.

Taken together, these results with derivatives of the sulfur-containing class of DBM substrate analogs have considerably advanced our understanding of the mechanisms by which they exert their antihypertensive activity. While these studies are not as yet complete, we feel that our hypothesized mechanism of action -- adrenergic uptake, neurotransmitter vesicular uptake, DBM oxygenation to form sulfoxide and exocytosis of NE now diluted by the presence of receptor-inactive sulfoxide in response to nerve firing -- is on much firmer ground than it was at the start of the current project period.

Selenium-Containing Compounds: Turnover-dependent Cofactor Depletion

During the current project period, we completed a thorough characterization of, and published (20,22,23), the unique enzymological characteristics of DBM-catalyzed turnover of our prototype selenide, phenyl-2-aminoethyl selenide (PAESe). We first confirmed that PAESe is indeed an excellent substrate for DBM. Enzymatic oxygenation produces phenyl-2-aminoethyl selenoxide (PAESeO), with kinetic parameters comparable to those for oxygenation of other active substrates -- e.g., the cognate sulfide, phenyl-2-aminoethylsulfide. As expected, oxygenation of PAESe by DBM follows the normal reductive oxygenation pathway; i.e., it exhibits absolute requirement for oxygen and electron donor, the 1:2:1 oxygen:electron:product stoichiometry diagnostic for monooxygenases, and stimulation by the DBM activator, fumarate.

As mentioned in BACKGROUND, what is remarkable about the processing of PAESe by DBM is that the enzymatic product, PAESeO, possesses a redox capability unlike that of any other DBM product. PAESeO reacts non-enzymatically with reduced ascorbate, giving back one molecule of PAESe and one molecule of fully oxidized ascorbate. During this current period we thoroughly characterized this non-enzymatic recycling reaction,and showed that it is a facile, stoichiometric process. Thus, we have clearly shown in *in-vitro* experiments that once enzymatic processing of PAESe by DBM begins, newly formed PAESeO is recycled back to PAESe, and this cyclic oxidation/reduction pathway continues until reduced ascorbate is depleted. Since reduced ascorbate is an essential cofactor for DBM, this *in-situ* depletion of reduced ascorbate prematurely terminates enzymatic activity.

We next turned to experiments in chromaffin vesicle ghosts in order to examine the consequences of this selenoxide recycling on ascorbate levels in the presence of the b-561-dependent ascorbate recycling system. We viewed such experiments as crucial, since while our solution results certainly demonstrated novel biochemistry for the selenide substrate, a physiologically meaningful rationale for drug design can only be established by demonstrating such $ASCH_2$ depletion even in the presence of the physiological $ASCH_2$ recycling system. Our expectation was that turnover of our selenides could well "short circuit" the b-561 system, since our *in vitro* work had revealed that no trappable semidehydroascorbate is formed during the nonenzymatic reaction of selenoxide and ascorbate. This was a highly significant finding since it is now generally accepted that semidehydroascorbate is the immediate product of DBM turnover, and it is this species which is recycled by b-561 at the expense of extravesicular ascorbate. We reasoned that if indeed the selenoxide/ascorbate redox reaction is a two-electron process in a kinetic sense (i.e., occurs without significant buildup of semidehydroascorbate), then accumulation of fully-oxidized ascorbate in the vesicle would occur as selenide is processed. Since fully oxidized ascorbate cannot be re-reduced by b-561, the net result of selenide oxygenation is thus the effective depletion of an essential cofactor for NE production. [It is noteworthy that the oxidation of catechols by selenoxides is normally viewed as a two-electron oxidation process in organic chemistry (24,25).]

The results of our chromaffin vesicle ghost experiments were fully consistent with this expectation (20,23). PAESe indeed causes a turnover-dependent depletion of reduced ascorbate in fully competent vesicle ghosts; as expected, this does not occur with the analogous sulfide substrate, where the sulfoxide product -- which is non-reactive toward ascorbate --builds up with time. Significantly, these experiments also established that the selenides are actively transported across the vesicle membrane, and are readily oxygenated by the membrane-bound DBM present in the vesicles. Under conditions of our experiments, vesicular reduced ascorbic acid stores were reduced by about 60%, and NE production was greatly reduced. Thus, our selenides exhibited precisely the effects we had predicted from our solution studies. A further observation was that the competition exhibited by PAESe against DA (the physiological substrate) for DBM oxygenation allowed the buildup of significant concentrations of DA within the

vesicles. This finding might contribute to the potency of these compounds, since DA is known to cause a powerful vaso-dilatory effect via direct DA-1 receptor activation.

In a thorough pharmacological bioassay study with the selenides, which was published within the past few months (26), we have clearly documented the potent antihypertensive activity of PAESe, the prototypic derivative of this series. We followed groups of SHR (control and drug-treated) over a two-week or more period, with dosing on a daily basis and with measurement of blood pressure, heart rate, and body weight over the entire period. In addition, we determined catecholamine levels in hearts of both control and experimental animals at the end of the experimental period. The data shows that PAESe exhibits a marked, dose-dependent antihypertensive effect in SHR, with as much as a 50% reduction in blood pressure beginning ca. 48 hrs. after the first dose and extending for more than two weeks. We found that the marked antihypertensive activity of PAESe in SHR occurs concomitant with a reduction in heart rate, heart weight, and heart catecholamine stores. An initial, short duration indirect sympathomimetic effect also occurs which is blocked by cocaine, thus suggesting that adrenergic neuronal uptake does occur through the same mechanism as that determined for the sulfur-containing compounds.

Taken together, we view our results with PAESe as illustrative of a novel strategy for designing modulators targeted at an enzyme such as DBM. The strategy entails design of an alternate substrate such that it will be readily converted by the target enzyme to a product, which is then capable of causing local depletion of an essential cofactor for the target enzyme itself. Such a strategy of *turnover-dependent cofactor depletion* is conceptually quite distinct from outright inhibition of the enzyme of interest, and might represent an especially useful approach to modulating compartmentalized cofactor-dependent enzymes for therapeutic purposes.

Finally, it is important to note that potential pharmacological applications of selenium-containing compounds must take into account any possible toxicological effects. We report in our J.PharmExpTher report (26) that there was no discernable effect on serum enzymes, electrolytes, or circulating metabolites in test animals treated chronically with large doses of PAESe. We see no indications at all of the characteristic hepatotoxicity patterns reported for inorganic selenium toxicity (27).

In summary, we feel that our hypothesized mechanism of action -- adrenergic uptake, neurotransmitter vesicular uptake, DBM selenoxygenation, ascorbate recycling and the consequent lowering of endogenous NE levels -- is on much firmer ground than it was at the start of the current project period.

N-Dealkylation, Olefin Oxygenation, and Mechanism-Based Inactivators

As mentioned in BACKGROUND, we have discovered that DBM readily catalyzes oxidative N-dealkylation of N-phenylethylenediamine (PEDA) and its derivatives [e.g. N-methyl-N-phenylethylenediamine (N-MePEDA]. During the current project period we carried out a thorough mechanistic study of the novel N-dealkylation activity for DBM (28,29) [We had discovered this reaction at the very end of the previous project period.]

The products of PEDA N-dealkylation were quantitatively identified as aniline (or N-methylaniline for N-MePEDA) and 2-aminoacetaldehyde, the latter compound being successfully trapped using NaBH4 reduction followed by N-succinimidyl p-nitrophenylacetate (SNPA) derivatization, and identified by HPLC and mass spectroscopy. In contrast, ether analogs of PEDA, i.e., phenyl-2-aminoethylether (PAEE) and its p-hydroxy derivative (p-OHPAEE), as well as 2-phenoxycyclopropylamine, are not substrates but are competitive inhibitors. Furthermore, 2-methyl-2-(anilino)-1-aminoethane (b-MePEDA) did not exhibit measurable substrate activity with DBM, in contrast to the excellent substrate activity of sulfur analog of b-MePEDA, 2-methyl-2-(phenylthio)-1-aminoethane (b-MePAES). DBM is inactivated during the N-dealkylation reaction in a time- and concentration-dependent manner, a phenomenon that has not, to our knowledge, been observed for any other oxygenase-catalyzed N-dealkylation reaction. Both PEDA and N-MePEDA, as well as b-MePEDA, inactivate DBM under turnover conditions. The inactivation exhibited pseudo-first order saturable kinetics and expected protection by the DBM substrate, tyramine. No reappearance of enzyme activity was observed after extensive dialysis. Radioactive labeling experiments with ring-tritiated PEDA showed incorporation of non-dialyzable radioactivity into DBM in the expected amount, consistent with covalent attachment of a reactive species derived from PEDA to the

DBM active site during enzyme inactivation. Although, aniline, N-ethylaniline, N-(2-fluoroethyl)aniline, m- and p-anisidine, p-toluidine and 5-hydroxyindole were found not to exhibit detectable DBM substrate activity, all of these inactivated the enzyme under turnover conditions. The isotope effect on partition ratio measured for di-deuterated PEDA was found to be a reflection of an isotope effect on Vmax and not on kinact.

Our results provide a strong support for the conclusion that DBM processing of PEDA and its cognates involves initial formation of a nitrogen cation radical, and it is this species which partitions between turnover and on the one hand or mechanism-based enzyme inactivation on the other. Results with ring-deuterated and ring-tritiated PEDA revealed that the amount of radioactivity incorporated into covalently-inactivated DBM by ring-tritiated PEDA is in agreement with that expected for covalent attachment of the para carbon to the protein. An 18O labeling study was carried out to test for oxygen rebound into the aminoacetaldehyde product, and the results demonstrated that the aldehyde oxygen of enzymatically-produced 2-aminoacetaldehyde exchanges very rapidly with solvent water, in agreement with literature reports.

Based on our mechanistic studies with heteroatom and olefinic substrates, we proposed a unified mechanism for all DBM-catalyzed monooxygenations, which is discussed in detail in papers published during the current grant period (28,29). Briefly, benzylic oxygenation by DBM proceeds through the unified intermediacy of a species with cationic and/or radical character when either methylene, olefin, carbinol, sulfur, or nitrogen is in the benzylic position. Oxygen transfer from copper to this species occurs readily in the case of carbon; similarly, the strong tendency of the sulfur cation radical towards sulfoxidation produces solely the sulfoxide and not the S-dealkylated product. For nitrogen, N-dealkylation of the cation radical species is a favored process and occurs readily. Thus, in essence, inactivation by PEDA- and PAME- type compounds represents a trapping of cation radical species formed along the N-dealkylation or olefin oxygenation pathway. Consistent with this mechanism, DBM is unable to carry out the oxidative O-dealkylation of phenylaminoethyl ethers under the usual reaction conditions, even though these bind very well to the active site and are competitive inhibitors for the enzyme. We have designed other novel substrate types for trapping such species and hope to examine them in detail during the coming grant period.

In bioassay work with PEDA which is still ongoing (30), we have observed several characteristics of PEDA's hypotensive activity which are quite distinct from that of the sulfides and selenides. First, we have observed not only depletion of NE and EPI, but also a significant rise in heart DA levels. Secondly, PEDA's hypotensive activity is much more prolonged, extending for six to seven days after drug withdrawal. Clearly, we are most interested in establishing that these differences do, in fact arise from a suicide inactivation mechanism being operative with PEDA, something we plan to explore in the coming project period.

Turning to our olefinic compounds, our laboratory was the first to demonstrate (in 1983 during our first project period; ref 14) that properly-designed olefins -- such as our prototype 1-phenyl-1-aminomethylethene (PAME) -- are epoxidized readily by DBM, that these are potent suicide inhibitors for DBM, and that mechanism based inactivation arises from a species with radical and or cationic character along the epoxidation pathway (14,29). Other groups in both academia and the pharmaceutical industry have made analogs of such olefins and some of these are very facile suicide inactivators indeed. We have examined and characterized kinetically and mechanistically a host of olefins based on the PAME structure; we now have in hand optimized olefinic inhibitors, some of which are <u>single turnover inactivators</u> for DBM (i.e. have a partition ratio of unity).(31). We are particularly anxious to explore the pharmacological properties of such compounds.

In a direction which we did not anticipate, we have found a number of non-aromatic substrates which are very readily oxygenated by DBM (31). Moreover, we have discovered an entirely new oxygenative competence for DBM with non-conjugated olefins -- the ability of DBM to readily carry out allylic hydroxylation. In a communication in press in J.Am.Chem.Soc (32) we report this first example of allylic oxygenation using the substrate 2-(1-cyclohexenyl)ethylamine, we demonstrate that this reaction is highly facile and stereoselective, we determine the *absolute* configuration of the enzymatically-produced

alcohol using a novel chiral resolving agent developed in our laboratory(33) together with nmr analysis of the diastereomeric adducts using the Mosher model (see refs 32 and 33 in Appendix)

Amine-Terminus-Modified Compounds.

During this project period, we designed several substrate analogs in which the primary amino terminus of the alkyl chain is replaced by a nitrogen-containing heterocyclic nucleus (34). For example, we demonstrated that 4-hydroxy-benzylimidazole (4-HOBI) is a facile substrate for DBM oxygenation. The enzymatic oxygenation of 4-HOBI results in oxygenolytic cleavage to produce 4-hydroxybenzaldehyde and imidazole. In contrast, when the terminal amino is replaced with pyrazole, a functionality which is uncharged at the pH of the DBM reaction and which is known to be a good copper ligand, the analog possesses potent DBM competitive inhibitory activity. Evidence in hand is suggestive that the pyrazole is interacting with the copper at the active site of DBM. We consider these results to have important implications to the future design of DBM-activatable "pro-drugs" possessing amino-terminal heterocycles which may impart adrenergic receptor agonistic or antagonistic activity to the enzymatic products.

We have also synthesized several phenylethyl alcohols and phenyl-ethanol sulfides (the non-amino-containing alcoholic analogs of phenethylamine and PAES, respectively). We have recently found these compounds to be appreciable enzymatic substrates only in the absence of fumarate. Fumarate, as well as other di-carboxylic acids, are known stimulators of DBM catalysis. The fact that substrate analogs possessing a primary alcohol in place of the normal positively-charged, primary amino terminus exhibit substrate activity only in the absence of fumarate suggests that fumarate is intimately involved in substrate binding of positively charged substrates.

Unsuccessful Work and Unfinished Business.

Although we feel that we have made good progress during the current project period, and in several instances have exceeded our own expectations, there are some areas in which we were less successful.

We have not extended our animal studies as much as we had planned with suicide substrates. We made the decision that we wanted to complete kinetic characterization of these classes to select those which would have the most promising from pharmacological potential; this we have now done and we will focus on animal and cellular level studies with these compounds during the coming year.

We have been unsuccessful in finding substrate activity with carboxyl-group-containing analogs designed to explore the "salt bridge" theory of the activating effect of fumarate (see pp. 38-39). Either our substrate analog designs were inappropriate, or the basic idea was erroneous. After considering the comments of the study section, we also decided not to pursue substrate analogs of the fused-ring type or those containing the pyridynyl nuclei.

Finally, we have not proceeded very far with the CNS-targeted pro-pro-drugs we had planned to examine. First of all, we have had considerable difficulty in the preparation of the (S,S) diastereomer which would be the most important of the four diastereomers. Secondly, as was noted in the Study Section review, there was a fairly tenuous argument for their utility. However, pharmacological research on central adrenergic reflex pathways which has progressed over the past few years may re-kindled our interest in this approach in the future.

D. METHODS

Selenium-Containing Compounds.

The unique aspect of this class of compounds arises from our hypothesis that they cause modulation of adrenergic neuronal activity through the novel strategy of turnover-dependent *local* depletion of the essential DBM cofactor, reduced ascorbate ($ASCH_2$). Because of this unique mechanism of $ASCH_2$ depletion, we recognize that compounds of this class may have wide ranging applications in neuroscience, in addition to their potential as hypotensive agents. Therefore, it is critical that we confirm

our hypothesized mechanism of action of the selenides using increasingly demanding criteria, and that we demonstrate this process directly in cell culture. There are three aspects to our proposed work:

A. Cultured Cell Experiments.

Our finding in chromaffin granule ghosts that PAESe depletes intravesicular $ASCH_2$, even in the presence of the b-561-mediated ascorbate recycling system, must be expanded to a full characterization of this process -- and its consequences on local and cytosolic $ASCH_2$ levels -- in functioning cells in culture. We have already obtained preliminary data confirming uptake of HOPAESe (as well as HOPAES) into cultured adrenergic neuronal cells (see PROGRESS REPORT). We have also established a viable tissue culture laboratory here, and have established reliable conditions under which both media ascorbate and oxygen levels can be accurately maintained and measured during the period necessary for experimentation.

In these experiments we want to establish three things. First, we want to characterize the time course of selenide uptake. Next, we want to show that selenides do, in fact, get further localized into the chromaffin vesicles within the cells, and determine the extent of such localization. Third, we want to demonstrate that it is indeed *local* $ASCH_2$ -- i.e. within the vesicles where DBM turnover occurs -- which is depleted by the selenides. This latter point is a subtle but important one, since cytosolic $ASCH_2$ does serve as the electron shuttle for reduction of the b-561 located in the vesicle membrane, with cytosolic ascorbate cycling between $ASCH_2$ and semidehydroascorbate and electrons ultimately coming from NADH by way of semidehydroascorbate reductase. (More complete discussion is in Appended papers #2,3,6,8). It is *within* the vesicles where we expect newly-formed selenoxide to react readily with *intra*vesicular $ASCH_2$ to form "dead-end" fully oxidized ascorbate. We must determine to what extent these expectations are borne out in functioning, cultured cells.

We plan to utilize the well-established PC-12 pheochromocytoma cell line in these investigations. PC-12 is an ideal neuronal model for these studies since the day-to-day maintenance of this well-characterized cell line is both much cheaper and much less time-consuming than the primary adrenal chromaffin cell cultures with which we had originally planned to work. Furthermore, PC-12 cells have been shown to possess the requisite enzymes of NE synthesis, including DBM (35), require ascorbate for maximal NE synthesis (21), and are homogeneous, producing only NE (unlike the heterogeneous primary cultures which are composed of both NE- and EPI-synthesizing cells). In order to characterize the time dependence of selenide uptake, and determine its subcellular localization after uptake, we will first utilize tritiated HOPAESe, which we have found to be the selenide which is most easily ring tritiated in our hands [We describe our tritiation procedure in detail in Ref 28]. In addition, by employing the very new technique of ICP/MS with the help of our collaborator, Professor Richard Browner (See Collab Arrangements), we anticipate also being able to quantitate picomole (per ml; i.e.nM) quantities of any selenium-containing compound, such as the refined derivatives noted below, without the necessity of tritiation.

Our typical protocol will be to add various concentrations of selenide to ca. 1×10^6 cells in multiwell plates, and after an appropriate incubation, stop the reaction by removal of the media, washing with ice-cold blank media, and cell harvesting. Since we have developed HLPC procedures for the separation of the test compounds from catecholamines and other cellular constituents (see appended reprints), we will be able to easily identify and quantify (by scintillation counting or ICP/MS) both the original selenide and any radiolabeled (or Se-containing, when we use ICP/MS)) metabolites. Uptake will be characterized as to the effects of pretreatment of the cells with, for example, pharmacologically-significant concentrations of cocaine, a known $uptake_1$ blocker, or in Na deficient media; this will correlate the characteristics of cellular uptake with results from whole-animal pharmacological experiments we have already performed. Analogous experiments wherein cell collection is followed by subcellular fractionation will be done to determine the intracellular localization of selenides after uptake. This is easily performed since the use of differential centrifugative procedures and enzyme markers for isolated subcellular organelles have been long-established in these PC-12 preparations. Entirely analogous experiments will be performed to examine the extent and localization of $ASCH_2$ depletion. As detailed in our recent JBiolChem papers (refs 19, 20; see Appendix) we have developed the requisite procedures for accurate quantitation of intra- and extra-vesicular $ASCH_2$ levels, as well as for measurement of DBM activity, in the isolated fractions.

Finally, in similar experiments in PC-12 cells, we will use the information gained in the above studies on the time course of uptake and maximal ascorbate depletion to "preload" cells with the test compound in order to demonstrate pharmacologically-significant exocytotic release of the intravesicular contents from these cells. Thus, stimulus of pre-loaded cells with the proper levels of acetylcholine or nicotine will be followed by sampling of the bathing media and quantitation of the released test compounds and metabolites by HPLC/scintillation counting. In addition, the ability of the test compound to compete with DA, the immediate precursor to NE, for cellular uptake, vesicular uptake, and DBM oxygenation will be testable using the same procedure (with the analysis of released NE) and will be highly instructive in validating our proposed mechanism.

B. Stereochemical Correlation and "Fine Tuning" of Ascorbate-Depletion.

We propose to utilize two approaches to manipulation of selenide-dependent $ASCH_2$ depletion in order to: a) further establish the absolute dependence of this process on DBM-catalyzed turnover; and b) to probe whether this process can be manipulated by judicious choice of the particular selenide substrate. In the first approach, we will take advantage of the enantiomeric selectivity of DBM for (S)-alpha-methylated derivatives of selenides (i.e. S-MePAESe) -- an unexpected enantioselectivity which we discovered last year. The second approach will make entail synthesis of, and biochemical characterization of ascorbate depletion by, more refined derivatives of PAESe.

We will perform these studies using bovine adrenal chromaffin granule ghosts, a preparation with which we now have considerable experience. These ghosts are an ideal model for adrenergic neurotransmitter synthesis and storage since they possess membrane-bound DBM, the catecholamine uptake transporter, the H^+-translocating ATPase, and most importantly, the cytochrome b_{561} ascorbate-dependent electron transporter. Using the methods we have published (19,20,23) we will be able to measure vesicular uptake and concentration, measure DBM turnover, and measure the effects of these compounds on intravesicular ascorbate levels.

In the experiments with the enantiomers of MePAESe, we will be looking for a sharp contrast in the behavior of the S vs R enantiomer; only the former should be processed by DBM within the ghosts leading to $ASCH_2$ depletion, while with the R enantiomer -- which we now know has virtually no substrate activity with DBM -- no such depletion should occur. We think that there is a good chance that both enantiomers will be taken up readily into ghosts, since we there are suggestions in the literature that *vesicular* uptake is much less discriminating toward stereoisomers than *cellular* uptake into the chromaffin cell. [Note that S-MePAES is stereotopically analogous to the naturally occurring a-substituted biogenic amines like (+)-amphetamine]. However, even if uptake discriminates between these enantiomers, we can still do these experiments by simply opening and resealing the ghosts in the presence of the selenides to initiate the experiments, something which we now have much experience. In fact, it is *precisely for this reason that we plan to do these experiments in ghosts* as opposed to PC-12 cells; ghosts are readily opened and resealed by us and many others in the literature for the purpose of tailoring their internal contents. Our protocols here for incubation, turnover, analysis of intravesicular $ASCH_2$, etc are straightforward following exactly our published procedures. Success in showing a direct correlation between the enantioselectivities of enzymatic turnover and $ASCH_2$ depletion even in the presence of the ghosts' fully functional b_{561} recycling system will represent very strong evidence for a direct link between these processes. [We have in hand quantitities of enantiomerically pure R and S MePAESe, both from stereospecic synthesis and from diatereomeric HPLC resolutions using our novel chiral derivatizing agent (33)].

The ability of diphenylselenoxide to act as mild oxidizing agents has been explored recently by synthetic organic chemists. It has been demonstrated that while the electron donating substituents in the aromatic ring will decrease the oxidizing power and generate more selective oxidizing agents, electron withdrawing groups yield more powerful, less selective oxidizing agents (36). We will therefore compare both para-F-PAESe and para-HO-PAESe as DBM substrates and as turnover-dependent ascorbate depletors both in vitro and in ghosts. [We have pHOPAESe in hand, synthesized from pMeOphenylmagnesium bromide plus metallic selenium to give p-MeOphenylselenol, followed by our standard oxazoline reaction and finally acid hydrolysis of the acetamide and deblocking with BBR3; p-

FPAESe will be synthesized using the same synthetic pathway]. We know that p-HOPAESe is an excellent DBM substrate; we anticipate p-FPAESe will be a slower substrate based on our kinetic studies on a host of ring substituted PAES derivatives which we carried published in 1981. On the other hand, the p-HOPAESeO sulfoxide product should be a poorer $ASCH_2$ recycler than p-FPAESeO. What will be the net kinetic effect on $ASCH_2$ levels both under in vitro assay conditions and in ghosts? We would like to know whether we can, in fact, manipulate turnover-dependent recycling -- in a kinetic sense --by judicious choice of the particular selenide substrate. This would be very useful for many future applications of these selenide compounds to modulate adrenergic outflow.

C. Determinations of Effective Tissue Concentrations.

We wish to correlate pharmacological data on the effect of selenides on isolated tissues -- which is being obtained by our collaborators at the University of Georgia (Drs. Hartle and Tackett; see Collaborative Arrangements) -- with our whole animal data on the hypotensive activity of the selenides. For this purpose, it is essential for us to carry out a limited investigation of the tissue concentrations of PAESe reached in both acute and chronic dosing in SHR. The methodology we will employ in these studies is that of straightforward pharmacodynamic investigations, and we will use the ICP/MS technique which allows the determination of picomole quantities of selenium-containing compounds. Thus, spontaneously hypertensive rats (SHR) will be dosed either acutely or chronically with doses of PAESe (and/or more interesting derivatives) and sacrificed at an appropriate time. Samples of heart, kidney, adrenal, brain, and blood plasma will be collected and analyzed for the content of PAESe and its metabolites. Of particular interest will be the concentration of PAESe in blood plasma, since it is the question of effective concentration of these compounds at adrenergic receptors which we want to know (the majority of adrenergic receptors responsible for the maintenance of blood pressure are in direct contact with blood plasma).

Sulfur-Containing Compounds.

This class represents our simplest strategic approach to the modulation of adrenergic neuronal activity. We plan to utilize our extensive understanding of the enzymology and pharmacology of these sulfur-containing substrates in limited experiments which parallel very closely those just described for the selenide class. Briefly, the three aspects of our proposed work are:

A. Cultured Cell Experiments: Using PC-12 pheochromocytoma cells, we will examine the cellular uptake, vesicular uptake, enzymatic sulfoxidation, and exocytotic release of a HOPAES (which we have found can be most easily tritiated), in cultured cells and subcellular fractions. In these experiments, note that we will be able to *directly measure the product sulfoxide, HOPAESO, unlike the situation in the case of the selenium-containing analogs where no appreciable product buildup occurs due to rapid back reaction with* $ASCH_2$. Note also that the sulfides are not expected to cause depletion of *intra*vesicular ascorbate, since the sulfoxides do not oxidize $ASCH_2$ to "dead-end" fully oxidized ascorbate. We have clearly demonstrated these two sharp distinctions between the sulfides and selenides in chromaffin ghost experiments (Ref 15, 20; Appended papers #1 & 3), which we consider hallmarks distinguishing their respective mechanisms of action, and confirmation in functioning cells is of major importance in confirming our hypotheses.

B. Pharmacodynamic Investigations: As discussed in PROGRESS REPORT, we now have in hand data on the anorexigenic effect of only the non-hydroxylated PAES derivatives, which supports our design hypothesis that HOPAES and HOMePAES have a much reduced ability to cross the blood brain barrier. We plan to confirm this contention by administering radiolabeled HOPAES and HOMePAES i.p. to SHR at doses which we have found cause appreciable antihypertensive effects, and then determining organ and tissue distributions after sacrifice. We feel that these experiments, though laborious, are quite important, since a primarily *peripheral* locus of action is an important facet of our proposed mechanism, and confirmation of minimal CNS access is an important consideration in any eventual clinical application of hydroxy derivatives of many of our compounds.

C. Stereochemical Selectivity: Here, once again, we will examine the correlation between the enantioselectivity of DBM sulfoxidation for only S-MePAES with the effects of R- vs S-MePAES in SHR, in PC-12 cells and in granule ghosts in order determine at the cellular and subcellular level whether or not enantiomeric specificity affects binding, uptake, and hypotensive activity in whole-animals. As is

the case with the Me-selenides, we have in hand quantities of enantiomerically pure R and S MePAES, both from stereospecific synthesis and from diastereomeric HPLC resolutions using our novel chiral derivatizing agent (33).

Optimized Suicide Substrates: Bioassay, Cellular and Subcellular Evaluations

Most of our detailed bioassay and subcellular work to date has focused on our *substrate* analogs for DBM. Over the past project period, we have examined and characterized -- kinetically and mechanistically -- numerous derivatives of two basic classes of novel mechanism-based DBM inactivators: olefins based on the PAME structure, and N-dealkylation inhibitors based on the PEDA structure (See Progress report; refs 28,29; Appended papers #7,10). We wish to emphasize that we have maintained in these inhibitors the structural features necessary for solubility and uptake; this reflects our desire to evaluate our inhibitors for pharmacological potential, not just for mechanistic purposes.

In the case of olefins, we have found that out of numerous PAME derivatives, para-F-PAME is an active, *single turnover inactivators* for DBM (i.e. it has a partition ratio of unity).(31). In the case of PEDA derivatives, beta-Me-PEDA is also an active mechanism-based inactivator with a very low partition ratio (about 50). We will therefore begin with these two compounds and evaluate them at the animal, cellular and subcellular levels using our usual protocols.

In preliminary bioassay work with PEDA (30), we have observed several characteristics of PEDA's hypotensive activity which are quite distinct from that of the sulfides and selenides. First, we have observed not only depletion of NE and EPI, but also a significant rise in heart DA levels. Secondly, PEDA's hypotensive activity is much more prolonged, extending for six to seven days after drug withdrawal. Clearly, we are most interested in establishing that these differences do, in fact, arise from a suicide inactivation mechanism being operative, and thus we are particularly anxious to examine b-MePEDA and p-F-PAME. As we have done with our sulfides and selenides, companion experiments will examine the uptake, enzymatic oxygenation, and inactivation kinetics of each derivative in chromaffin granule ghosts.

The contrast between the magnitudes and time courses of the antihypertensive activities (and the attendant changes in NE tissue levels) for compounds designed to *inactivate* DBM in comparison to the effects already observed for the *substrate-analog* classes will allow us to *critically assess the ultimate value of either approach*. Similarly, the contrast between the subcellular organellar effects of the mechanism-based inactivators vs. the substrate analogs will allow us to assess and compare their neurochemical characteristics. We therefore feel that the results of these comparative studies will be of considerable value.

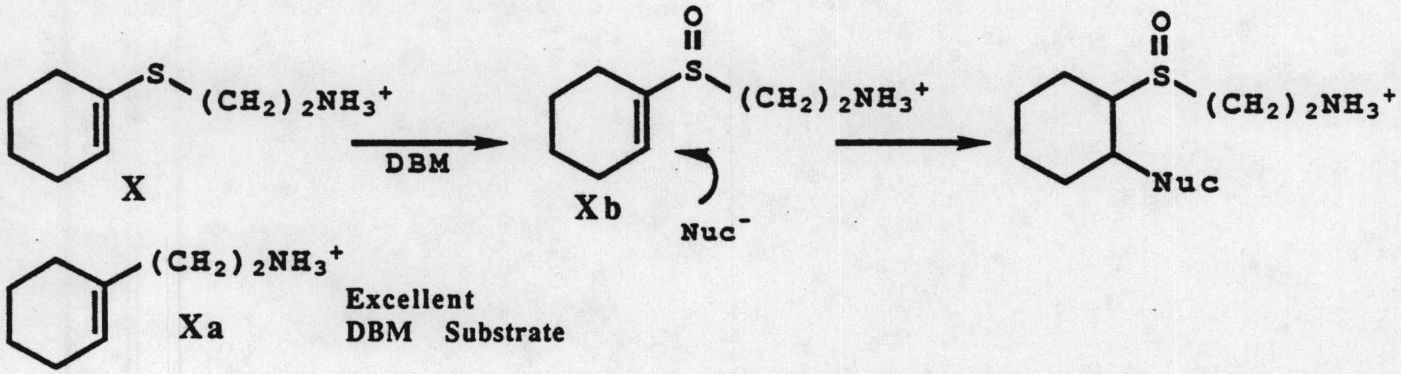
New Compounds

In this subsection, we propose to synthesize and examine new classes of DBM-targeted compounds. We will first examine their detailed enzymology, including kinetic evaluations, determinations of the turnover-dependence and reversibility or irreversibility of inhibition, etc. For those compounds whose structural features, chemical properties and enzymological parameters suggest that they would be promising candidates for bioassay, we will use our standard SHR antihypertensive assay method. Compounds which exhibit appreciable activities in these assays would then be examined at the cellular and subcellular levels.

A. Inhibitors Designed to Generate Michael Acceptors Upon DBM Monooxygenation:

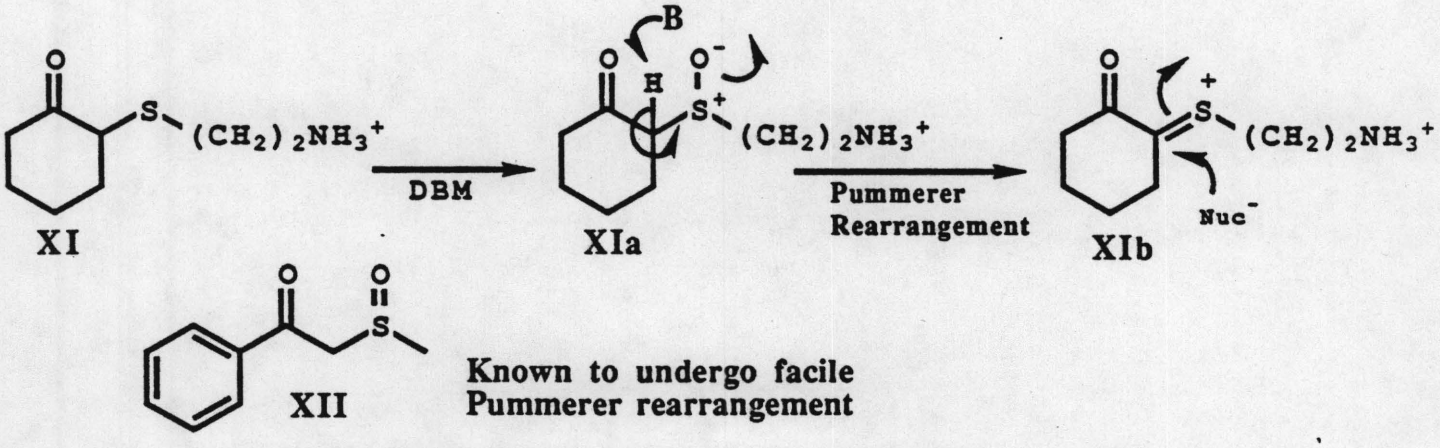
In our previous studies we have demonstrated that the sulfur analog of phenylethylamine, PAES, and its ring and side chain substituted compounds are excellent substrates for DBM, and the products have been identified as the corresponding sulfoxides (10,15). In addition, our recent work has established that compound Xa, in which the aromatic moiety of the phenylethylamine has been replaced with a cyclohexene, is an excellent substrate for DBM with the steady state kinetic parameters comparable to that of the best DBM substrate, tyramine (32). Therefore, it is likely that sulfur containing compounds such as X could be good substrates for DBM, and DBM monooxygenation will result in the generation of a

corresponding sulfoxide (Xa) in the DBM active site. It is well known that alpha, beta unsaturated sulfoxides are extremely reactive towards nucleophiles and undergo Michael type addition reactions under mild conditions. Since DBM monooxygenation of X generates a highly reactive alpha, beta unsaturated sulfoxide (Xb) in the active site of the enzyme, X could be a potential turnover-dependent irreversible inactivator for DBM as shown in the scheme below, if an active site nucleophilic moiety is properly oriented for covalent attachment. These compounds will be synthesized by alkylating the potassium salt of thiocyclohexanone with 2-bromoethylalcohol (37) and then converting the corresponding alcohol to the terminal amine by standard techniques. If we encounter any problems in the above synthesis (thiones are sometimes difficult to work with) the following synthesis will be used. Cyclohexanone will be reacted with two equivalents of 2-aminoethylmercaptan and the product (the thioacetal) will be oxidized with one equivalent of sodium periodate to yield the desired product. Depending on the outcome of our inhibition experiments with X, we will also examine similar selenium containing compounds as potential inhibitors for DBM.



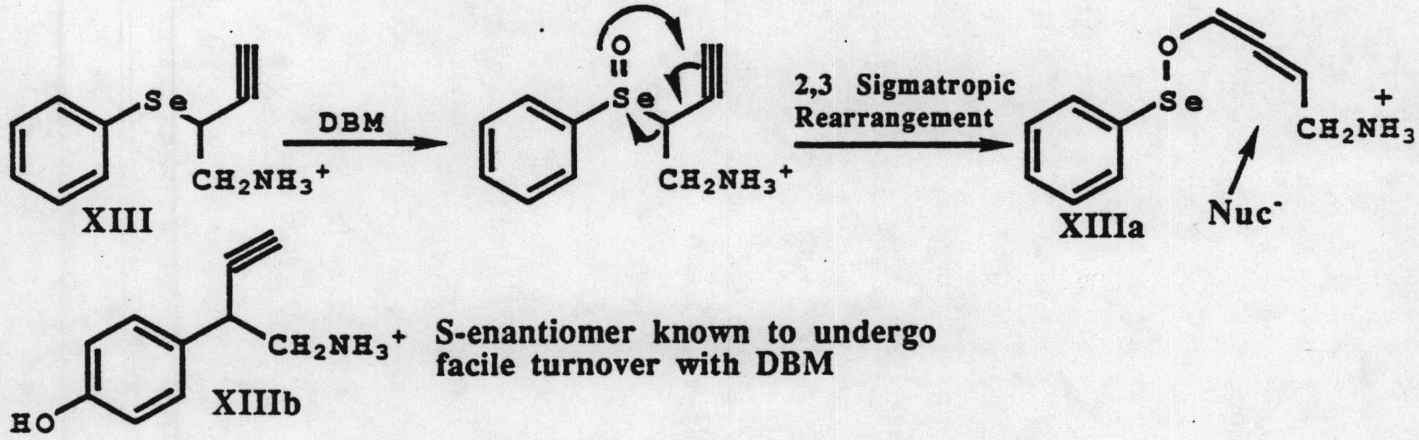
B. Inhibitors Designed to Undergo Pummerer Type Rearrangement Upon DBM Mediated Sulfoxidation.

We have recently discovered that properly designed aliphatic sulfides possessing an aminoethyl moiety are good substrates for DBM [for example both butyl 2-aminoethyl sulfide as well as cyclopentyl 2-aminoethylsulfides are good substrates for DBM (38)]. In addition, as noted above, a cyclohexene ring confers excellent substrate activity for DBM. Therefore, it is likely that compounds such as XI will be substrates for DBM. If these compounds are substrates for the enzyme, the DBM-mediated monooxygenation should yield the corresponding sulfoxide product (XIa) similar to the case of the respective aromatic cognates or aliphatic sulfides. Due to the presence of a carbonyl group at the beta position, the alpha hydrogen of the sulfoxide XIa is acidic which facilitates the "Pummerer rearrangement". [Compounds bearing an acidic hydrogen alpha to a sulfoxide functionality, are known to undergo Pummerer rearrangement under mild conditions; for example, the beta-keto sulfoxide XII undergoes Pummerer rearrangement with aqueous acids or other acidic reagents at room temperature to form the hemimercaptal of an alpha-keto aldehyde (39)]. As shown in the scheme below, Pummerer rearrangement of the sulfoxide product XIa will result in an extremely reactive species (XIb) which could be attacked by a side chain nucleophile in the active site of the enzyme to inactivate the enzyme irreversibly. We note however that such a rearrangement will be dependent on the presence of a properly oriented basic residue in the enzyme active site to abstract the acidic alpha hydrogen. Compound XI will be synthesized by treating commercially available 2-chlorocyclohexanone with 2-mercaptoethanol under basic conditions and then converting the terminal hydroxyl group to an amine by standard techniques.



C. Inhibitors Designed to Undergo [2,3] Sigmatropic Rearrangement:

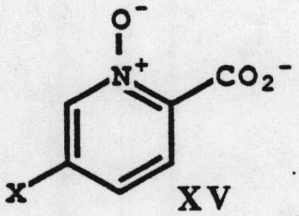
Our work has amply demonstrated the ability of DBM to carry out facile selenoxidation of PAESe and its derivatives (22). Recent work by Walsh et al has demonstrated that flavin enzyme, cyclohexanone oxygenase, is also capable of mediating selenoxidation, and that enzymatically generated propargylic and allylic selenoxides readily undergo [2,3] sigmatropic rearrangement in the active site of the enzyme (40). Based on this precedent, we will examine compound XIII as an initial test for DBM-mediated potential [2,3] sigmatropic rearrangements. As shown in the scheme, compound XIII should readily undergo [2,3] sigmatropic rearrangement upon selenoxidation, to generate a highly reactive species, allenic selenic ester (XIIIa), capable of leading to enzyme inactivation. We anticipate that compounds such as XIII should be good DBM substrates based on the following two reasons: a) Our own work has established that cognates of both PAESe and PAES which contain substituents in the alkyl side chain are *still excellent* substrates for DBM; b) Substitution of an acetylenic group at the benzylic position of the "classical" DBM substrate, tyramine, does not abolish the substrate activity (S-enantiomer of compound XIIIb is a substrate for DBM); in fact, it *increases* the affinity for binding significantly (41). Compound XIII will be synthesized by treating the phenyl methanesulfonate of the corresponding N-protected amino alcohol with sodium phenyl selenate. If we encounter problems due to the competing S_N2' addition of phenylselenide to the acetylenic moiety in the above synthesis, we will use trimethylsilyl blocked sodium acetilide instead of sodium acetilide and this group will be removed at the last step by treating with F^-. The N-protected amino alcohol, 2-hydroxy-4-acetamido-1-butyne will be prepared by treating the 3-acetamido propanal with sodium acetylide in liquid ammonia according to the procedure by Billimoria and Maclagan (42).



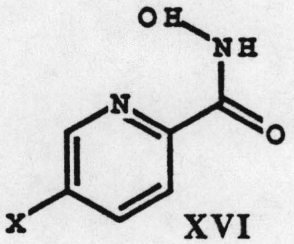
D. Picolinic Acid Based Inhibitors for DBM:

5-Alkyl picolinic acids are long-known to be potent inhibitors for DBM, and the most potent of all, fusaric acid [5-butyl picolinic acid, (XIV)], is known to exhibit potent antihypertensive activity (43) Although a complete characterization of inhibition of DBM by picolinic acid has never been reported, the existing evidence suggests that the inhibition is due to the chelation of active site Cu (43). We will synthesize and examine a series of derivatives of 5-substituted picolinic acid that are designed to be better Cu(II) chelators than fusaric acid itself. N-oxides and ketoximes are better ligands for copper than

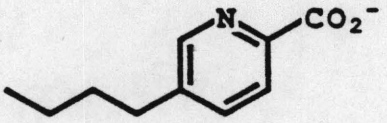
pyridine nitrogen or carboxylic acids, respectively (44). We therefore will examine compounds XV and XVI as potential inhibitors for DBM. The results obtained with these compounds may be valuable in designing more potent and specific inhibitors for DBM, and they may also help reveal structural information about the DBM active site Cu. These compounds will be synthesized from commercially available substituted picolinic acid derivatives by standard synthetic methods. For example, the hydroxylamine derivative XVI will be synthesized by simple nucleophilic displacement reactions of easily accessible ester derivatives of substituted picolinic acids with hydroxyl amine. The N-oxide XV will be synthesized by H_2O_2 oxidation of fusaric acid under mild conditions.



XV



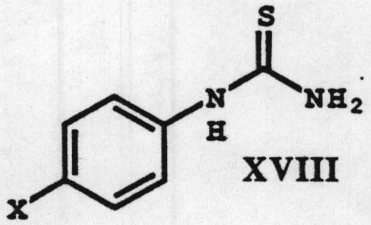
XVI



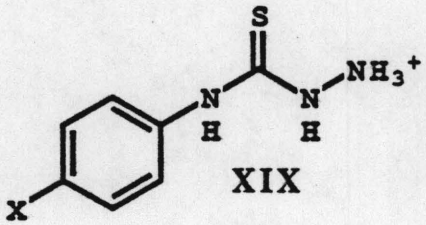
XIV
Fusaric Acid

E Other Inhibitors:

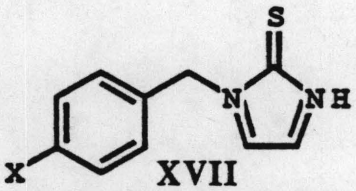
Recently, Kruse et al have demonstrated that 1-(4-hydroxyphenyl)imidazole-2-thiol (XVII) is an extremely potent competitive inhibitor for DBM (45). They suggested that the potent inhibition is due to the ability of XVII to act as a multisubstrate mimic of the binding of oxygen and phenylethylamine to the reduced enzyme. Based on the above precedent, compounds such as XVIII-XX may be potent inhibitors for DBM. Due to the flexibility of the side chain and the presence of a free terminal amino group in these compounds, it is possible that these compounds may be even better reversible inhibitors for DBM than XVII. In addition, compounds XXI and XXII are designed to test whether the positioning of a strong copper ligand such as hydroxylamine or thiol in the hydroxylation site will lead to the enzyme inactivation by tightly interacting with the active site copper(s) of DBM. While, compounds XVIII and XIX are commercially available, N-hydroxy N-phenylethylenediamine will be synthesized by reacting O-alkoxy aniline derivatives with N-protected 2-bromoethylamine and then, acid deblocking of both protecting groups. Compound XXII will be synthesized by the treatment of 2-chloro-2-phenylethylamine with Na_2SO_3 followed by acid hydrolysis (46).



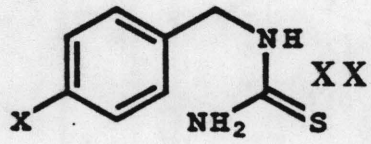
XVIII



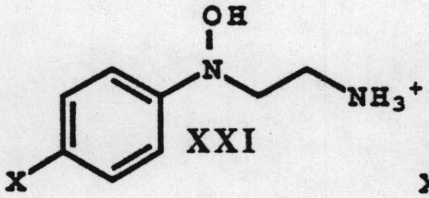
XIX



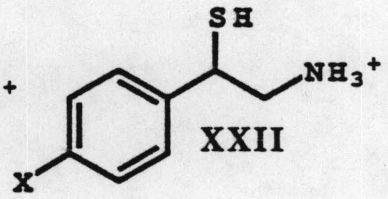
XVII
Potent DBM inhibitor



XX



XXI



XXII

Prototype Inhibitors Targeted at Tyrosine Hydroxylase

Our basic approach to date has obviously been to design and evaluate novel compounds targeted at DBM. As detailed in BACKGROUND, our rationale for this approach is recognition that DBM is conveniently sequestered together with its b-561 ascorbate recycling system within catecholamine vesicles, that our compounds are effectively transported to the target DBM via presynaptic uptake and vesiculation, and that DBM should be relatively insensitive to down regulation.

During the next project period we wish to carry out a very limited pilot evaluation of one class of compounds targeted at the enzyme tyrosine hydroxylase (TH), the monooxygenase which catalyzes hydroxylation of Tyr to dihydroxyphenylalanine (DOPA), thus sitting two steps ahead of DBM in the pathway leading from the essential amino acid, Phe, to the neurotransmitter, NE. Ever since the early literature on catecholamine biosynthesis, the TH-catalyzed reaction has been regarded as the putative rate limiting step (7), although, as pointed out later (8), there may be a shift toward rate determining DA hydroxylation under certain metabolic conditions. For our purposes, we are particularly interested in contrasting the behavior of a potent TH inhibitor with those of our DBH-targeted compounds. This would provide reference data by which to evaluate the relative advantages of targeting two different points along the catecholamine pathway and would provide some indication of the importance of the sequestering and uptake accessibility of DBM in terms of rationale drug design. Also, if TH is indeed the rate limiting step, then inhibiting TH may well have a much more drastic effect on NE levels than would inhibition of DBM; thus we may obtain insight into the consequences of "DBM-targeted modulation" vs more drastic "TH-targeted cutoff" on NE production.

One finds statements in the literature and in textbooks with an almost anecdotal quality that TH inhibition is not a fruitful approach to antihypertensive agents. [See, for example in Goodman and Gilman, 6thEd., page 72: "Unfortunately inhibition of this enzyme is not of therapeutic benefit...." and references cited]. After perusal of the literature we contend that a careful in vitro study directly demonstrating that a particular compound is a potent TH inhibitor, and characterizing its effect on NE production is badly needed. The results of such a study, when combined with the reports of others on the regulation of TH and its pharmacological consequences, will be very valuable in evaluating the validity of conclusions regarding TH-targeting. This is our rationale for proposing our limited pilot study.

In recent work in our laboratory with the enzyme phenylalanine hydroxylase (PAH) we discovered that PAH readily carries out oxygenation of 4-pyridylalanine to the corresponding 4-pyridylalanine-N-Oxide (53). In the course of this work, we synthesized and fully characterized both various pyridylalanines and their corresponding N-oxides. We reason there is an excellent chance that TH will similarly carry out conversion of 4-hydroxy-3-pyridylalanine (which exists as its pyridone tautomer) to the corresponding N-oxide (Both PAH and TH are non-heme-iron monooxygenases). This N-oxidation would generate the corresponding pyridone-N-oxide as the product, which is actually a hydroxamic acid N-oxide, *an excellent ligand for iron!* Thus, the product could readily inhibit TH by ligating to the essential Fe atom in the active site. We will therefore carry out a limited pilot project to see if this approach to TH inhibition works under standard assay conditions. (TH will be isolated in our laboratory from PC-12 cells, see ref 54). We will carry out in-vitro enzymology, and if this idea works we will carry out studies in chromaffin granule ghosts to contrast the effects on NE production with the effects of our DBM inhibitors. No SHR work is planned at this stage since the amino acid structure of our inhibitors ensures that there will be central effects that we are not prepared to sort out at this stage.

DBM Mechanism

Our ongoing work on this project has certainly made contributions to the mechanistic enzymology of DBM, a subject of much interest in a number of laboratories. Two particular studies will be carried out primarily for the information they can provide about the structure and function of DBM. These entail demonstrating DBM-catalyzed aromatization of compounds designed to trap reaction transients generated by single electron transfer; and active site peptide mapping with one of our suicide inhibitors which is ideal for this purpose.

We have very recently observed that DBM catalyzes aromatization of 1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA) a process which proceeds via the normal DBM reductive monooxygenation pathway. We find this observation very exciting, since aromatization of cyclohexadienes represents a well established trapping reaction diagnostic of single electron transfer processes. As set out in our publications, our unified mechanism for DBM processing postulates initial single electron transfer from substrate; thus, for example, we visualize that heteroatom cation radicals are initially formed with our N,S or Se substrates.

We will determine the kinetic characteristics of CHDEA turnover, and unequivocally prove that the only product from the CHDEA/DBM reaction is 2-phenylethylamine; to date we have no evidence for any oxygenated products being formed. ^{1}H-NMR, ^{2}H-NMR and GC-MS analysis of the product from paired enzymatic reactions, carried out either in H_2O or in $^{2}H_2O$, will demonstrated whether deuterium incorporation into the product, 2-phenylethylamine, occurs during CHDEA/DBM turnover. Dideuterated CHDEA (CHDEA-d_2) will also be prepared and examined as a substrate for DBM. The NMR and GC-MS analysis of the product from CHDEA-d_2/DBM reaction will reveal whether loss of deuterium at the benzylic position of the product occurs. If aromatization of CHDEA during DBM turnover indeed arises solely from initial single electron transfer from the cyclohexadiene moiety to the activated copper-oxygen specie -- and not from a methylene hydroxylation pathway -- then no deuterium incorporation or loss will be seen in these two experiments, respectively. Thus, lack of deuterium loss (or incorporation) would effectively rule out any mechanism involving initial hydroxylation at the exocyclic methylene followed by aromatization in DBM/CHDEA reaction, but would be consistent with initial abstraction of H from a ring methylene. Since aromatization of cyclohexadienes represents a well established trapping reaction diagnostic of single electron transfer processes, these experiments may provide direct support for the ability of the activated copper oxygen species of DBM to carry out a single electron transfer process.

Recently, several attempts have been made to map the active site of DBM by using active site directed radiolabeled suicide inhibitors. (49). However, results of these experiments have not been successful due to the non-specific labeling of a large number of peptides in the tryptic map. In our view, a major problem with these previous studies is that the inhibitors used do not possess a terminal amine functionality. Our work with non-amine containing compounds clearly indicates to us that such compounds interact "loosely" with the active site of DBM (51). Therefore, it is possible that the nonspecific labeling of several peptides may be a result of "movement" of the enzyme-activated species within the active site of the enzyme during the inactivation process. In our previous studies, we have developed several classes of terminal-amine-containing suicide inhibitors for DBM (29). With both PAME and PEDA (our olefinic and N-dealkylating classes), we have extensively characterized the mechanism of inhibition. We have developed the methodology for, and successfully carried out, of ring tritiated N-phenylethylenediamine (PEDA) with high specific activity, in our laboratory and shown that a stoichiometrically consistent amount of radioactivity is incorporated into the enzyme during the inactivation (28). Similar results have been obtained with tritiated PAME. We therefore propose to use tritiated PEDA, (we already have ca. 300 mg in hand) to map the active site of DBM. Since PEDA is a potent suicide inhibitor for DBM and structurally very similar to the "regular" substrate, phenylethylamine, we expect that we will not encounter the complications which have plagued previous attempts by others. Since the complete amino acid sequence of DBM is now known (52), we will be able to compare our results with sequence data for confirmation.

F. VERTEBRATE ANIMALS

All of the animal experiments which are described in this proposal will begin at Mercer University School of Pharmacy under the direction of Dr. Herman, who has experience in managing animal populations. At Mercer, we have contracted for the use of a well-equipped modern animal care facility which includes caging designed for dog, guinea-pig, rabbit, rat, and mouse populations. This facility was especially designed for animal care and has all of the pertinent sanitary features, including separate rooms for food storage and preparation, surgical appliances, and disposal equipment including an incinerator for sacrificed animals. We have contracted for the use of these facilities for the past six years, and are assured that this arrangement will continue for the foreseeable future. We will house a population of both SHR and WKY rats (Charles River) at Mercer. We hereby affirm that we will conform to the guidelines suggested for the use of experimental animals outlined in "Guiding Principles in the Care and Use of Animals" (publ. by the Council of the American Physiological Society), the "Principles for the Use of Animals" (OPRR), and the "Guide for the Care and Use of Laboratory Animals" (OPPR) in the proposed investigations.

There are two types of animal experiments proposed here, one involving SHRs and one involving normal rats. Test animals -- both normal (WKY) and SHR (CRL:CD9SD/BR) rats -- will be purchased from a reputable dealer (Charles River), housed in approved caging for a maximum of a few weeks, and used for bp measurements as outlined in the body of the proposal. Animals will be sacrificed by cervical dislocation, and sacrificed animals will be incinerated.

The proposed experiments involved a relatively small number of animals. We expect to use approximately 100 SH rats and 100 normal rats. All rats will be male, 9-13 weeks old, purchased in batches of 20 from a national supplier (currently Charles River). We believe that these experiments are quite necessary to the goals of this research, and that our studies will benefit the health of man, the welfare of human society and the progress of knowledge. This proposal has been reviewed and approved by the Animal Welfare Committee (letter attached).

G. COLLABORATORS

We will collaborate with Professor Richard Browner of the School of Chemistry and Biochemistry of Georgia Institute of Technology for the purpose of applying the exciting new technique of ICP/MS to the detection and quantitation of our selenides and their metabolites in biological samples. Professor Browner possesses one of a total of only four such units in use in academic laboratories in North America, and his group has pioneered research in sample introduction approaches for ultra micro trace analysis. With their instrument, they will be able to measure selenium at levels of 10 nano-molar or better which is more than enough sensitivity for our purposes. As explained in the body of the proposal, this technique will be of particular value in our PC-12 experiments and in our pharmacodynamic experiment with our selenides.

We will collaborate with two pharmacologists at the School of Pharmacy of the University of Georgia -- Drs Randall Tackett and Diane Hartle. Drs. Tackett and Hartle have already obtained used their sophisticated computerized hemodynamic instrumentation to clearly confirm the antihypertensive activity of PAESe. Pharmacological experiments to probe cardiovascular effects of our selenides on isolated blood vessels are in progress. They have also initiated similar studies with PEDA. These investigations represent the final link in the chain of experimentation on which we have embarked -- now spanning from enzyme to ghosts to cells to isolated vessels to whole animals. In addition, Drs. Tackett and Hartle have agreed that due to unique biochemical mechanisms operative with our compounds, they are highly interesting to them as neurochemical probes. We are looking forward to further collaboration along these lines in the future.

Dr. Richard Mailman of the University of North Carolina has initiated a collaboration with us to evaluate the effects of our DBH-directed compounds in Brindled Mottled Mutant mice. We anticipate that the unique biochemical properties of our compounds will enable us to obtain important information about the relationship between DBH activity and the development of noradrenergic innervation. This is an important frontier research area in neuroscience.

We will continue our relationship with Professor Leon Goldberg, Chairman of the Committee on Clinical Pharmacology at the University of Chicago. SWM and one of the project personnel have visited Dr. Goldberg's laboratory, and we have had a number of discussions regarding the cardiovascular pharmacology of our compounds in light of our bioassay results. Dr. Goldberg is also very interested in the unique properties of our compounds because interfering with DBM may lead to a buildup of DA, a molecule known to have vasodilator action, particularly in the renal and mesenteric vasculature. This is an area in Professor Goldberg is an acknowledged international authority. While we have now developed considerable expertise in carrying out bioassays for cardiovascular activities of our compounds and in probing the pharmacological mechanisms operative, Dr. Goldberg has kindly consented to serve both as an expert consultant in cardiovascular pharmacology, and as a collaborator in further evaluating those compounds of interest from his clinical perspective.